Original Article

On the in vitro anti-myeloma effect and mechanism of Andrographolide through PI3K/AKT/mTOR and AMPK/mTOR dual signaling pathways

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Abstract: Objective: To investigate the effects of Andrographolide on the proliferation, apoptosis, and cell cycle arresting of MM cells. The effects of Andrographolide on PI3K/AKT/mTOR and AMPK/mTOR dual signaling pathways were inquired. The specific molecular mechanism of Andrographolide inhibiting myeloma cell proliferation was explored as well. Methods: MTT assay was applied to determine the effects of Andrographolide on the proliferation of myeloma cell lines. The effects of Andrographolide on cell cycle and apoptosis were detected by flow cytometry. Western blot and flow cytometry were used to detect the apoptosis-related proteins and cyclin-associated proteins in both monotherapy and combination therapy. Western blot method was employed to measure the changes of IGF-IR/PI3K/AKT/mTOR and AMPK/mTOR signaling pathways.

Results: The experiment found that Andrographolide could inhibit the proliferation of MM cells. And flow cytometry results showed that Andrographolide could induce MM cell apoptosis and cell cycle arrest in G1 phase. Western blot results exhibited that, the expressions of pro-apoptosis-related protein such as PARP-1, caspase-3, caspase-9, and Bak, as well as G1 phase arresting protein P21 were all activated by Andrographolide, while the expressions of anti-apoptosis protein such as Mcl-1, HIAP-1, and survivin, plus the pro-G1-associated proteins such as CDK4 and CDK6 were all inhibited. Conclusion: The inhibitory effect of Andrographolide may be through the IGF-IR/PI3K/AKT/mTOR signaling pathway, but the administration of IGF-I can reverse the anti-myeloma effect of Andrographolide.

Keywords: Andrographolide, multiple myeloma, apoptosis, cell cycle arrest, PI3K/AKT/mTOR, AMPK/mTOR dual signaling pathways

Introduction

Multiple myeloma (MM) is a malignant clonal cell-borne disease originating from B lymphoblastic cell lines of the germinal center, characterizing abnormal production of monoclonal immunoglobulin light chains [1, 2]. Although the body shift implantation and the use of new therapeutic drugs have significantly elevated the MM remission rate, MM is still considered to be incurable [3]. At present, studies on MM pathogenesis mainly focused on cytogenetic abnormalities, and the “co-evolution” of MM and the microenvironment bone marrow stromal cells, which caused the sustained activation of survival signal and drug-resistance within MM cell. Progress has been made, but MM is still incurable [4, 5]. Therefore, it is clinically urgent to study the relevant mechanism of MM cell growth to look for new treatment strategy.

The risk of tumor in diabetes is believed to be connected with the presence of insulin resistance in diabetic patients. Insulin resistance leads to an increase in insulin and insulin-like growth factor (I/IGF), which is mitogenic and capable of binding to its receptor, thus activating phosphatidylinositol 3-kinase/protein kinase B signaling pathway and promoting cell proliferation and the occurrence of tumor. I/IGF is considered to be an important link between diabetes and tumors [6-8]. Therefore, tumor incidence risk may be lowered through reducing hyperinsulinemia in diabetic patients.

Andrographolide is heat-curing and detoxifying, anti-inflammatory and analgesic, and also effective in treating tumor. The aim of this study is to investigate the role of Andrographolide in myeloma and to explore the molecular mechanism of anti-myeloma cell proliferation through
Andrographolide through PI3K/AKT/mTOR and AMPK/mTOR dual signaling pathways, and to provide a theoretical basis for future myeloma treatment.

Method

Cell culture

MM cells, taken out from the liquid nitrogen, were then put into 37°C water bath, and agitated to accelerate the melting process. After complete melt, the cells were transferred to a sterile centrifuge tube that contains 5 ml culture medium. The centrifugation was conducted at 800 rpm for 5 min. The supernatant was discarded. The cells were re-suspended in fresh medium and then transferred to a culture flask, which was then put into an incubator at 37°C with 5% CO₂. The fluid was changed for every other day. MM cell lines were cultured in RPMI1640 medium containing 10% FBS, then put into a saturated humidity incubator under 37°C with 5% CO₂.

MTT assay to detect cell proliferation

MM cells in logarithmic growth phase were harvested. The cells were centrifuged at 800 rpm for 5 min. The supernatant was abandoned and the cells were re-suspended in RPMI1640 medium containing 10% FBS. After cell number counting, the cell density was adjusted to 5×10⁴/ml. The cells were seeded into a 96-well plate, with 200 μl in each well. Drugs with different concentrations (10 μM, 20 μM, 40 μM, and 80 μM) were added, while the control group was added with PBS/DMSO in all 4 wells. The cells were cultured for 24 hours. Each well was added with 20 μl MTT, and the plate was put into an incubator with 5% CO₂ under 37°C for 4 hours. The absorbance value at 570 nm was measured under a Bio-Rad meter. The experiment was repeated for three times.

Flow cytometry to detect cell cycle

The cells were seeded into a 6-well plate, which was then added with drugs of different concentrations (10 μM, 20 μM, 40 μM, and 80 μM) respectively. After 24 hours, the cells were washed with PBS and digested with 0.25% trypsin. The cell suspension was collected and centrifuged at 800 rpm for 5 min. The supernatant was abandoned and the cells were re-suspended with PBS. After another centrifugation, the supernatant was abandoned and the cells were re-suspended again. The single-cell suspension density was adjusted to 2×10⁶/ml. The suspension was added into 95% ethanol at 4°C, three times of the volume, making the final fixed concentration 70%. After slightly agitated, the suspension was immediately put under 4°C condition for more than 24 hours. The cell proliferation index (PI) was calculated, where PI (%) = (S + G2 - M)/(G1 + S + G2 - M) ×100%.

Annexin V-FITC/PI double labeling method to detect apoptosis

The cells in the 6-well plate were cultured for 24 h, and the supernatant was collected into a centrifuge tube. The cells were digested with 0.25% trypsin without EDTA. After centrifugation at 2000 rpm for 5 min, the supernatant was discarded. The cells were washed with PBS twice, then centrifuged at 2000 rpm for 5 min, and the cell concentration was adjusted to 5×10⁵-1×10⁶/ml. A total of 1 ml cells was obtained, then centrifuged at 1000 rpm for 10 min under 4°C. The supernatant was abandoned. The cells were added with 1 ml cold PBS, and gently mixed to make the cells suspend. Again the cells were centrifuged at 1000 rpm for 10 min under 4°C, then the supernatant was discarded. The above step was repeated for 3-4 times. The cells were re-suspended in 200 μl Binding Buffer. Following this, the cells were added with 10 μl Annexin V-FITC, and mixed gently, then put in darkness under room temperature for 1 min. After adding 300 μl Binding Buffer, and then 5 μl PI, the apoptosis rate was measured.

Western blot to detect the expressions of caspase 3, caspase 9, PARP-1, Bak, cyclinD1, CDK4, CDK6, p21, IGF-IR, PI3K, AKT, PAKT, mTOR, p70S6K, and 4E-BP1

MM cells from each group were collected and were pretreated with IGF-I and LY294002 for 1 h, then treated with Andrographolide for 24 h. All the cells were washed with PBS twice. Each cell bottle was added with 400 μl lystate, then 40 μl PMSF at 10 mmol/L. The bottle was slightly agitated, and then placed on ice for 10 min for sufficient lysis. The cells were pipetted with a sterile syringe repeatedly. The lysis was added into an EP tube, which was ice-bathed for 30 min, and then centrifuged at 12000 g for
15 min. The supernatant was transferred to a new EP tube. Protein concentration was determined by ELISA method with a microplate reader. Each tube was added with 20 μl Buffer per 100 μl, boiled for 5 min, and mixed uniformly. The tubes were stored under -80°C.

The above samples were obtained to conduct electrophoresis in 12% SDS-PAGE. The separated protein bands were transferred to PVDF membrane by wet method and closed under room temperature for 1 h. The proteins were incubated with primary antibody (caspase 3, caspase 9, PARP-1, Bak, cyclinD1, CDK4, CDK6, p21, IGF-IR, PI3K, AKT, PAKT, mTOR, p70S6K, and 4E-BP1 at concentration 1:1000) under 4°C overnight. After been washed for three times, the proteins were incubated with secondary antibody (concentration 1:1000) for 1 h. Again, the proteins were washed with PBST for three times. After colored and fixed, the expressions of the above proteins were determined through chemiluminescence.

Statistical methods

All data are represented by mean and standard deviation. In the 10 μM, 20 μM, 40 μM, and 80 μM group were compared, repeated measurement data were analyzed by repeated measures. Each experiment was repeated for three times, using One-way ANOVA, in which P < 0.05 denoted statistical significance. All data were processed with the Graphpad Prism 5.0 (Graphpad Software. San Diego, CA).

Results

MTT test results

MTT results showed that Andrographolide can inhibit MM cell proliferation. Andrographolide significantly inhibited the proliferation of MM cell lines, in a dose-dependent manner, in which the highest 80 μM density showed the most obvious inhibitory effect. See Figure 1.

Flow cytometry results

The effect of Andrographolide on cell cycle was observed by flow cytometry after 24 hours' medication. It was found that drugs inhibited cell cycle progression. G0/G1 phase cells increased, while S phase cells decreased. Cells stagnated in the G0/G1 phase. There was a slight increase of G0/G1 phase cells, but not a significant decrease of S phase cells, indicating that cell proliferation was inhibited to a certain extent. As shown in Figure 2.

Annexin V-FITC/PI double labeling results

The cells were stained with Annexin V-FITC/PI and detected by flow cytometry after 24 hours' medication. The results exhibited that drugs of all concentration can induce the apoptosis of cancer cells in a proportional degree, with the highest 80 μM group the most significant effect. See Figure 3.

Western blot to detect the expressions of caspase-3, capase-9, PARP-1, cyclinD1, CDK4, CDK6 and p21

In order to verify that Andrographolide can induce apoptosis and cycle arrest of MM cells, changes of relative proteins in the apoptosis and cycle arrest were detected using Western blot method. The results showed that Andrographolide can induce apoptosis through the mitochondrial pathway, activate caspase-3, caspase-9 and PARP-1, and regulate proteins in G1 phase, in which the cyclinD1, CDK4 and CDK6 decreased, while p21 increased, suggesting that Andrographolide induced G1 phase arrest of MM cell lines. As shown in Figure 4.
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Figure 2. The effects of Andrographolide on cell cycle by flow cytometry. (Order is 0 μM, 10 μM, 20 μM, 40 μM, 80 μM).

Figure 3. The effects of Andrographolide on apoptosis using Annexin V-FITC/PI double labeling. (Order is 0 μM, 10 μM, 20 μM, 40 μM, 80 μM).

Western blot to detect the mechanism of Andrographolide

Andrographolide plays a hypoglycemic effect mainly through increasing insulin sensitivity, and reducing intestinal reabsorption of glucose, thereby reducing insulin level of blood circulation, and hyperinsulinemia of diabetic patients. Therefore, it is speculated that Andrographolide plays an anti-tumor effect throu-
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In order to further confirm that Andrographolide functions through PI3K/AKT/mTOR pathway, IGF-I (100 ng/ml) and PI3K inhibitor LY294002 (100 ng/ml) were added to activate and inhibit the pathway respectively, and the effects of Andrographolide (80 μM) on MM cell line apoptosis were observed, as well as the change of signaling pathway proteins. It was found that IGF-I can attenuate the apoptosis of MM cells induced by Andrographolide. The expressions of PI3K, pAKT and p70S6K were increased after IGF-I was added into MM cells, while adding LY294002 can inhibit the above proteins. Therefore, it is concluded that Andrographolide can inhibit MM cell proliferation via IGF-IR/PI3K/AKT/mTOR signaling pathways. As shown in Figure 6.

**Discussions**

In recent years, the coincidence of diabetes and tumor had attracted great interests among international researchers. A number of epidemiological data suggested that diabetic patients would suffer increased risk of tumor. Literatures reported that type II diabetic patients would suffer more risk of colorectal cancer [9]. A recent meta-analysis showed that the risk of developing breast cancer increased by 23% and colorectal cancer by 26% if the patients had suffered diabetes. Likewise, patients with diabetes and tumor will get higher rates of recurrence and mortality [10-12]. The relationship between diabetes and myeloma has also been paid with great attention.

The risk of tumor in diabetics is believed to be correlated with insulin resistance in diabetic patients. Insulin resistance leads to an increase of insulin and insulin-like growth factor (I/IGF), which can facilitate mitosis. I/IGF can bind to its receptors and activate phosphatidylinositol 3-kinase/Protein kinase B (Akt PDK/AKT) signaling pathways, thus leading to cell proliferation and tumor. In this way, I/IGF is considered to be an important bridge between diabetes and tumor [13]. Therefore, it may be effective to lower the risk of tumor incidence through reducing hyperinsulinemia in diabetic patients.

Cell cycle is an indispensable process of cell life, which is mainly regulated by cyclin-dependent kinase (CDK), and the activity of CDK is controlled by cyclin and CDK inhibitor (CDKI)
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Cyclin binds to CDK and regulates cell cycle positively, while CDKI binds to CDK and regulated cell cycle negatively. At present, cyclin D is considered to be the major protein than can positively regulate G1 phase, and bind with CDK4/CDK6. Cyclin A mainly regulates G2 phase, binds with CDK2, while cyclin B regulates M phase, and binds with CDK1 [16].

CDK1 has been identified to be composed of 7 family members, including p21, p27, p57, p15, p16, p18, and p21. It can bind with a variety of cyclin CDK complexes, in which the strongest bond is with CDK4/CDK6, and CDK1 is considered to be mainly in G1 phase. The present experiment showed that metformin could induce the G1 phase arrest in MM cells. Western blot analysis exhibited that p21 expression increased, while cyclinD1 expression decreased. The p21 and cyclinD1 were positive and negative regulating proteins in G1 phase respectively, and had caused the inhibition of CDK4 and CDK6, and the cell cycle arresting in G1 phase without entering into the S phase.

Tumor is considered to be cell apoptosis being blocked, suggesting that induction of cell apoptosis is an important method of treatment. Cell apoptosis is mainly mediated by the death of cell membrane (exogenous pathway) and mitochondria (endogenous pathway). Caspase cascade reaction is the basic process of cell apoptosis, of which caspase 3 mediates the common pathway, caspase 8 mediates exogenous pathway, and caspase 9 mediates the mitochondrial pathway respectively. Bcl-2 protein family is the major mitochondrial protein that regulates apoptosis, IAP (Inhibitor of Apoptosis Protein) is a naturally occurring inhibitor of caspase in the cell, while releasing the inhibition of IAP on caspase can induce cell apoptosis.

The experiment results showed that Andrographolide can induce the apoptosis of MM cell lines, accompanied by an increase of caspase 3, caspase 9 and PARP-1. MM cell proliferation is correlated with intracellular activation signaling pathway. Insulin and insulin-like growth factor (I/IGF) are the important growth factors that facilitate myeloma cell proliferation [17, 18]. MM cells overexpress their receptor IGF-IR [19], and the combination of them can activate the downstream signaling pathway PI3K/AKT/mTOR, as well as mediate MM cell proliferation and drug-resistance [20]. PI3K/AKT/mTOR is an important pathway to facilitate cell proliferation in myeloma, which is a major consensus in the treatment of myeloma. The mTOR exists in two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 downstream target proteins include S6 ribosomal protein kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E), thus releasing eIF4E, facilitating the transcription and translation of cyclinD1, HIF and c-myc, thus leading to cell proliferation and survival [21-23]. The mTORC2 protein can phosphorylate Ser473, activate protein kinase B (PKB/AKT), which can activate downstream mTOR through phosphorylating serine 2448 on mTOR, or indirectly activate mTOR through activating Rheb (Ras homolog-enriched in brain), thus promoting cell survival [24, 25].

It is found that Andrographolide can inhibit the expression of IGF-IR in MM cells, and the downstream PI3K, while the phosphorylation of AKT, mTOR, 4E-BP1 and the activation of p70S6K are also inhibited. To further verify the speculation, IGF-I and PI3K inhibitor were added, and the effects of Andrographolide on cell apoptosis signal protein molecules were observed through activating or inhibiting the pathway. It is found that, the addition of IGF-I can inhibit the cell apoptosis induced by Andrographolide, accompanied by the de-activation of PI3K, pAKT and p70S6K, and the activations were synergistically inhibited when adding LY294002.
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Disclosure of conflict of interest

None.

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References